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Bioassay of Major Fractions of Cigarette Smoke Condensate by an Accelerated Technic[1]

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SUMMARY

The results of bioassays of fractions of cigarette smoke condensate (CSC) using mice previously treated with 7,12-dimethylbenz[a]anthracene (DMBA) are reported. The assay was designed to detect complete carcinogens as well as fractions possessing only tumor-promoting activity. The results showed that the weak acid (phenol) fraction and two neutral fractions exhibited statistically significant activity. In addition, the third neutral fraction gave results suggesting activity, and the ether-soluble base fraction exhibited possible activity. In these experiments, the basic dose of each fraction was the equivalent of 94 to 375 mg of CSC per week. In contrast, mice not previously treated with DMBA are usually given much more CSC. In the present study, the positive fractions were clearly detected in 40 weeks. The assay system thus has merit as a monitor for the fractionation of CSC and other similarly complex carcinogenic materials.

INTRODUCTION

Although a number of assays of fractions of cigarette smoke condensate (CSC) have been reported, the tumorigenic activity of the crude material cannot be explained by the content of the purified substances thus far identified. Solution of this problem has been difficult because the biologic assays are laborious and extraordinarily expensive. The problem is further complicated by the fact that CSC is a very complex mixture (8) and is a relatively weak carcinogenic stimulus.

Wynder and associates (11–13) have shown that, on an activity per gram basis, certain neutral subfractions are the most active of those obtained. However, the extent to which these subfractions contribute to the total activity of CSC is not clear. Several years ago, one of us found that a heptane-soluble fraction (HSF) of CSC exhibited more carcinogenic activity (per cigarette basis) than the original CSC. Silicic acid chromatography of HSF gave a fraction eluting with benzene which contained 6% of crude CSC weight and more than 50% of crude CSC activity (2). Day (3) has shown that a neutral fraction exhibited about 80% as much tumorigenicity as crude CSC. These various neutral fractions represent the most refined CSC products that still retain the bulk of the original tumorigenicity.

The tumorigenic activity of CSC may be due to combined tumor-initiating and promoting activities (7, 11). Although the weakly acidic fraction appears to contribute substantially to the latter activity, the active neutral fractions may also play a role in this regard. Therefore, bioassay of CSC fractions by the system recommended by Poel (6) as an accelerated test for carcinogens would be of value. In this system, a subthreshold (initiating) dose of 7,12-dimethylbenz[a]anthracene (DMBA) is followed by successive doses of the prospective tumorigenic agent, and the results reflect combined initiating and promoting effects. Gellhorn (4) and Van Duuren *et al.* (6) have shown that crude CSC does show increased tumorigenicity using this type of bioassay system.

The present report concerns the relative tumorigenicity of fractions of CSC when tested in the concentrations equivalent to those found in crude CSC by the accelerated bioassay procedure. Also, data are presented on the losses in activity on recombining all fractions.

MATERIALS AND METHODS

Fractions of CSC prepared as described in a companion report (9) were shipped under Dry Ice by air express from Philadelphia to Buffalo at approximately monthly intervals. They were picked up immediately after arrival and were kept in the cold until diluted for experimental study. The diluted fractions were placed in brown glass bottles, each containing sufficient material for about 5 days of application, and were stored at −20°C until required. As needed, one bottle at a time was removed to the working refrigerator. The solvents for the 12 fractions, the crude tar, and the reconstituted crude tar were based on acetone, using water as required. Before adjusting the final volume, a few ml of each solution were diluted with an equal volume of water and were read in a pH meter. In several cases, the resulting solutions were either acidic or basic in comparison with the crude CSC. Those thought to contain substantial quantities of acid or base were therefore treated

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with either concentrated ammonium hydroxide or concentrated acetic acid to provide a final acidity more nearly like that of the starting material. The three "neutral" fractions were not adjusted to neutral pH, because, although acidic, they would be rapidly buffered by skin constituents.

The reconstituted fraction did not entirely dissolve in half of the desired volume of acetone but left an acetone-insoluble sediment. The sediment was treated with a small quantity of 50% aqueous acetone, in which it almost completely dissolved, and was then poured into the acetone solution, followed by the remaining acetone required to provide the final appropriate volume. The fractions were largely soluble in the solvent mixtures employed. Nevertheless, several of the fractions, as well as the crude and reconstituted CSC, probably existed, in part as a fine suspension; the solutions were somewhat turbid. At the beginning of the study, the bottles of working solution were shaken manually before use. Toward the end of the study, the bottles were placed in an ultrasonicator for better mixing prior to daily use.

The concentration of each fraction depended on the yield obtained on fractionation. The basic unit of concentration was the equivalent of 30% crude CSC, which in a pilot study was an effective tumor-promoting stimulus. All fractions but one were therefore diluted so that the concentration of each was equal to its concentration in a 30% solution of crude tar; e.g., if the recovery of Fraction X amounted to 100 gm per kg of crude tar, the concentration of X used for the assays would be 3%, because X was present at a concentration of 3% in a 30% solution of crude tar. In some cases, other dilutions were also employed to provide for semiquantitative comparison of the respective fractions. Thus the crude CSC was tested at concentrations of 30%, 15%, and 7.5%. The reconstituted material was applied in 2 concentrations equivalent to 30% and 15% CSC. Fractions 3, 4, 6, 7, 8, 9, and 11 were used at the standard equivalent of 30% CSC. Because Fraction 5 was toxic, it was applied only at an equivalent of 15% CSC. The neutral Fractions 12, 13, and 14 were expected to be of particular interest, and these were applied at concentrations equivalent to 60% and 30% CSC.

Female ICR Swiss mice were treated at 60 days of age with 125 μg of DMBA in 0.25 ml of acetone, and the dose was checked in a spectrophotometer. Before treatment and at monthly intervals after, the dorsal hair was removed with electric clippers. After 3 weeks, the animals were treated 5 times a week with the promoting stimulus, which consisted of 0.25 ml of one or another of the various fractions. Each experimental group consisted of 50 mice. Five control groups were established. The first consisted of 36 untreated animals. The second group contained 50 mice painted with DMBA followed by a promoting stimulus of 0.03% croton oil in acetone. This group served as a positive control to ensure that the response of the animals to the DMBA treatment was normal. The third group consisted of 43 mice treated a single time with DMBA; the fourth group consisted of 50 mice treated a single time with DMBA followed by a promoting stimulus of acetone only. These two groups served as negative controls to disclose any abnormal sensitivity of the animals to DMBA treatment. The fifth control group consisted of 44 animals treated only with acetone in place of DMBA and promoting stimulus. All of the animals were examined weekly, and the number and distribution of any tumors were noted. The experiment was terminated after 55 weeks of promoting stimuli, when the mice still alive were sacrificed.

During the course of the study, we questioned whether the water used in preparing the reconstituted CSC solutions might affect the biologic activity of this material. Solvents play a profound role in the penetration of hydrocarbon carcinogens into the skin (1). Furthermore, it was possible that the acetone-insoluble materials, which were more prominent in the reconstituted fraction than in the crude CSC, represented new tumor-promoting agents that were produced as an artifact of the isolation procedure. Accordingly, additional groups of 50 mice were pretreated with DMBA and then treated with 30% crude CSC, and the equivalent amount of the reconstituted fraction was dissolved only in acetone. The part of the reconstituted material which did not dissolve in acetone was discarded. Mice were painted for 11 weeks and were observed for an additional 4 weeks.

Four of the fractions, 3, 4, 7, and 9, were particularly insoluble, so it was possible that the test preparations were suspensions with only trace amounts in true solution. Under these circumstances, testing by skin application might be totally inadequate. Accordingly, these four fractions were tested additionally by subcutaneous injection. For this purpose, 0.3 gm of each of the 5th and 6th batches was ground in a mortar with 10 ml of trioctanoin until a homogeneous suspension was achieved. Beginning at 40 days of age, groups of 30 ICR Swiss female mice were injected intrascapularly twice a week for 5 weeks with 0.1 ml of freshly prepared suspension of one or the other fraction. A fifth group of 30 mice was injected with trioctanoin alone. As nearly as possible, the same site was employed for every injection. On one occasion, the test material was not freshly prepared but consisted of suspensions which were prepared earlier and preserved in the refrigerator. For another injection, sesame oil was the vehicle instead of trioctanoin. On both occasions, all of the experimental groups and the control group were treated similarly.

After 28 weeks, two animals from each group were sacrificed for routine examination. The remaining animals have been observed until dead or moribund or for a period of 66 weeks.

RESULTS AND DISCUSSION

No tumors appeared in 80 mice that were untreated or treated only with acetone. In 93 mice treated either with DMBA alone or with DMBA followed by acetone, 6 tumors appeared in 4 animals (Table 1). This result is typical of our experience with this dose of DMBA in ICR Swiss mice (Chart 1). We anticipate that approximately 0.8% of mice treated with DMBA will develop tumors after the equivalent of 36 weeks of promoting stimulus. In the present study, the first of 93 DMBA-treated mice developed tumors in the 36th week and the second in the 40th week. Of 50 mice painted with DMBA followed by croton oil, 38 developed a total of 225 tumors. Throughout the experiment, the numbers of tumors developed in these positive controls were within the 95% confidence limits of 17 other experiments using the same batch of croton oil. Thus the positive and negative controls behaved in a satisfac-

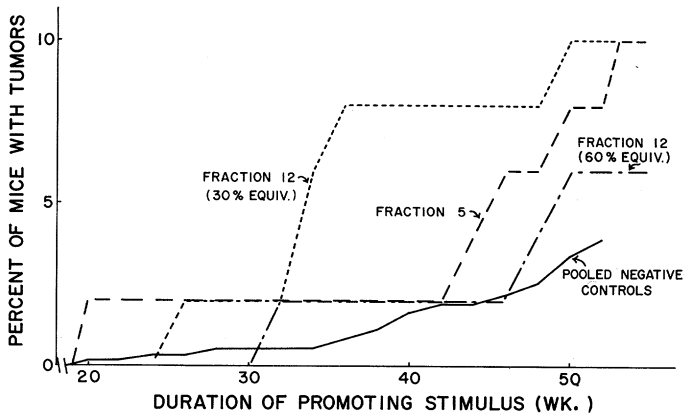

Chart 1. Tumor induction in mice treated with Fractions 5 or 12. The pooled negative controls consist of 899 mice treated with 125 μg 7,12-dimethylbenz[a]anthracene followed by acetone or with 125 μg 7,12-dimethylbenz[a]anthracene only in a series of 14 experiments.

tory way. The incidence of tumors in the experimental groups showed significant activity in the crude CSC, the reconstituted CSC, and in Fractions 8, 13, and 14 (Table 1). In addition, the behavior of mice painted with Fractions 12 or 5 was such, throughout the study, as to suggest that Fraction 12 was also active and that Fraction 5 was of possible activity (Chart 1). Although the first tumor in Group 5 appeared during the 20th week, the other 4 positive mice developed tumors only late in the experiment, at a time when DMBA-treated negative controls also developed tumors. The differences in mg/dose for the reconstituted CSC, crude CSC, and the sum of Fractions 1–14 in Table 1 are reflections of small weight losses occurring during fractionation (9).

In the repeat study using only the acetone-soluble portion of the reconstituted CSC, 8% of the mice treated with crude CSC and 10% of the mice treated with reconstituted CSC developed tumors after 11 weeks of painting and 4 additional weeks of observation. Accordingly, there was no indication that the activity of the reconstituted fraction was due to the use of the water or to the production of the components that proved to be insoluble in acetone after reconstitution.

No sarcomas could be confirmed in the animals that were injected subcutaneously with suspensions of the insoluble fractions and observed for 66 weeks. One animal from Fraction 3 had a subcutaneous growth at the injection site which was first noted in the 51st week. Unfortunately, the animal died under conditions such that microscopic examination of the tissue was impossible. Other tumors which appeared in the various groups included pulmonary adenomas, lymphomas, and mammary carcinomas, tumors which are not unusual in old untreated Swiss female mice. Every animal that was autopsied showed deposits of tarry material at the injection site. It is therefore probable that the adjacent tissues were exposed continuously to saturated solutions of these tarry materials in the extracellular fluid.

Table 1

Fraction no.	Group or fraction	Average mg per dose[a]	No. of mice[b]			Total no. of skin tumors
			Survivors	With tumors	With skin cancers	
	Untreated		30	0	0	
	Acetone only		34	0	0	
	DMBA only		31	2	0	3
	DMBA + acetone		37	2	0	3
	DMBA + croton oil		25	38	15	225
1A	Crude CSC	75	33	30	11	58
1B	Crude CSC	38	41	22	7	46
1C	Crude CSC	20	46	11	0	12
2A	Reconstituted CSC	65	35	28	11	71
2B	Reconstituted CSC	33	41	9	2	12
3	Bases before, insoluble	0.89	42	3	0	3
4	Bases after, insoluble	0.51	45	3	1	3
5	Bases, ether-soluble	2.3	30	5	3	6
6	Bases, water-soluble	1.8	36	1	1	1
7	Weak acids, insoluble	5.9	38	2	1	2
8	Weak acids, ether-soluble	7.5	34	19	7	48
9	Strong acids, insoluble	1.3	38	1	1	1
10	Strong acids, ether-soluble	2.2	44	2	0	2
11	Strong acids, water-soluble	27	41	2	0	2
12A	Neutrals, 80% methanol-soluble	7.6	45	3	0	4
12B	Neutrals, 80% methanol-soluble	3.8	43	5	2	5
13A	Neutrals, cyclohexane-soluble	24	41	8	1	8
13B	Neutrals, cyclohexane-soluble	12	30	7	1	7
14A	Neutrals, nitromethane-soluble	4.4	40	26	6	38
14B	Neutrals, nitromethane-soluble	2.2	37	13	6	15

Development of tumors in treated animals. CSC, cigarette smoke condensate; DMBA, 7,12-dimethylbenz[a]-anthracene.

[a] See test for concentrations in terms of "equivalent tar dose."

[b] After 55 weeks of test (65 weeks of age). Fifty mice per group except 36–44 in solvent, untreated, and DMBA controls.

Each mouse received the fractions from 0.8, 4, 8, or 10 gm of CSC. In comparison, of the mice painted with 8% CSC (5.2 gm over a period of 55 weeks), 22% developed local tumors even though much of the dose probably was not absorbed into the tissues. It is improbable, therefore, that the materials rendered insoluble by the isolation procedure and represented by Fractions 3, 4, 7, and 9 could account for an important part of the tumorigenic activity of the CSC.

On the basis of the results from Groups 1B, 1C, and 2B (Table 1), the reconstituted fraction appears to be somewhat less active than the crude material from which it was obtained. Comparison of 2A with 1B and 2B with 1C suggests that more than half of the activity remained in the reconstituted fraction. It thus seems unlikely that any major active fraction was lost in its entirety during the fractionation procedure. It may be concluded that Fractions 8, 13, and 14, and probably Fraction 12 and possibly Fraction 5, contained the major tumor-promoting substances. Of the neutral fractions, Fraction 14 was the most active. Fraction 8, the ether-soluble weak acid (phenolic) fraction, was a more active promoter than any of the individual neutral fractions. It is not possible, however, to determine whether Fraction 8 was equal in activity to the combined neutral fraction or to what extent removal of any of the individual fractions from CSC would have reduced the activity of the remainder.

Wynder and Hoffmann (12) found that, in addition to the phenolic, basic, and neutral fractions, the strong acid fraction also exhibited tumor-promoting activity. Indeed, the strong acids were relatively more active than the neutral fraction. In our hands, mice treated with the strong acid fractions, 9–11, developed fewer tumors than the controls. The difference between the results of Wynder and Hoffmann and those reported here may arise from slight differences in the separation procedure inasmuch as the phenols (weak acids) and strong acids appear in adjacent fractions. Conversely, the differences in findings may result from the dosage used in the tests. In the present study, each fraction was tested at the concentration at which it existed in 30% crude CSC solutions, so that its relative importance would be ascertained. Furthermore, in the present study, the acidic fraction was neutralized so that nonspecific effects of pH would not interfere with the interpretation of the results.

The present analysis was carried out for 55 weeks. Even so, except for the doubtful Fraction 5, the positive fractions were all identifiable at 40 weeks (Table 2). At that time, effective guidance for further fractionation was available in a relatively short time with a minimum requirement of CSC. In this study, most of the mice were treated with the equivalent of 375 mg of CSC weekly, and substantial numbers of tumors were produced by only 94 mg of crude CSC weekly. In the absence of DMBA, however, we have generally used the equivalent of 550 mg or more of CSC weekly (2, 5). Wynder and Hoffmann (12) used the equivalent of 1,000% CSC (yield = 0.5%; dose = 5.0%) as Fraction "BI" to obtain a significant tumor incidence. Day (3), using the equivalent of 300 mg of CSC a week as the neutral fraction, observed only 5.3% tumors in 52 weeks. The accelerated test procedure thus provided important savings in materials as a guide for systematic fractionation of CSC. It should be equally valuable for other complex materials with similar biologic action.

Table 2

Group	Equivalent CSC dose[a] (%)	Mice with skin tumors (%)	Total no. of skin tumors/ no. of mice
Combined DMBA-treated negative controls	0	2	0.02
Croton oil		64	4.2
1B	30	24	0.54
2B	30	42	0.84
8	30	14	0.22
12B	30	8	0.08
13B	30	6	0.06
14B	30	12	0.12
5	15	2	0.02

Animal results after 40 weeks. CSC, cigarette smoke condensate; DMBA, 7,12-dimethylbenz[a]anthracene.

[a]See Table 1 for mg/dose.

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